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Effect of RNAi on expression of ecdysis-related genes in silk glands of the silkworm, *Bombyx mori*

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Abstract: In insects, ecdysone signal transduction pathway is mediated by a cascade of transcription factors, and ecdysone receptor (EcR), Broad complex (BR-C) and E74A are believed to be the early response factors. To study the functions of these early transcription factors in Lepidoptera, double-stranded RNAs (dsRNAs) of these genes of *Bombyx mori* were synthesized *in vitro* and injected into the wandering larvae. Phenotype changes were observed and the expression of these and related genes were analyzed by quantitative real-time PCR (qPCR). Asynchronous pupation and eclosion, deviant wing and death of pupae and moths were observed after interference of *BmEcR*, *BmBR-C* and *BmE74A*. The qPCR results revealed that partial silence of *BmEcR* decreased the mRNA levels of *BmE74A*, *BmE74B*, *BmHR3A* and *BmβFTZ-F1*. Partial silence of *BmE74A* reduced the mRNA levels of *BmEcR*, *BmBR-C*, *BmE74B*, *BmHR3A* and *BmβFTZ-F1*, while partial silence of *BmBR-C* resulted in a significant decrease in the expression levels of all tested genes except for *BmIAP*. These results implied that *BmBR-C* may act at the earlier stage in ecdysone signal pathway and regulate the expression of other transcription factor genes as well as ecdysone receptor gene *BmEcR* in the silkworm.

Key words: *Bombyx mori*; RNA interference; metamorphosis; ecdysone; transcription factor

1 INTRODUCTION

In insects, apoptosis of the degenerating larval tissues, such as prothorax gland, salivary gland and fat body, is initiated by ecdysteroids during metamorphosis (Dai and Gilbert, 1997; Müller *et al.*, 2004; Silva-Zacarin *et al.*, 2007). The ecdysis-related transcription factor genes *E93*, *BR-C*, *E74A* and *βFTZ-F1* regulate the expression of the apoptosis-related caspase genes and the autophagy-related genes (*Atgs*) in the salivary gland in *Drosophila melanogaster* (Baehrecke, 2003; Martin and Baehreche, 2004). In the fat body of *D. melanogaster*, ecdysone initiated autophagy through PI3K and Tor signal transduction pathways and ecdysone receptor signals accelerated the autophagic process by down-regulating the PI3K pathway (Rusten *et al.*, 2004; Scott *et al.*, 2004).

In the silk gland of *B. mori*, expression of the ecdysone receptor gene *BmEcR* and the ecdysis-related transcription factor genes *BmBR-C* and *BmE74A* precedes that of the other ecdysis-related transcription factor genes *BmHR3A* (Eystathioy *et al.*, 2001), *BmHR39* (Niimi *et al.*, 1997), *BmFTZ-F1* (Sun *et al.*, 1994), *BmE74B* (Sekimoto *et al.*, 2007), *BmE75B* and *BmE75C* (Swevers *et al.*, 2002), indicating that *BmEcR*, *BmBR-C* and *BmE74A* might be at the up-stream of the ecdysone signal pathway. However, further experiments are needed to determine the relationship among the ecdysone-related receptors and transcription factors and their influence on the metamorphosis in *B. mori* (Li *et al.*, 2010).

In this study, effects of RNAi of the genes, *BmEcR*, *BmE74A* and *BmBR-C*, on the expression of the ecdysis-related transcription factor genes, including *BmHR3A*, *BmHR39*, *BmFTZ-F1*, *BmE74B* and *BmE75B*, were investigated and the possible relationship between the expression of these genes and phenotype changes during the metamorphosis from larva to adult was discussed.

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2 MATERIALS AND METHODS

2.1 Insects

Larvae of *B. mori* (strain 7532) were fed with mulberry leaves and reared at 25℃ and 70% relative humidity in the laboratory conditions until adults or to the stages used for experiment.

2.2 RNA interference of *BmEcR*, *BmBR-C* and *BmE74A*

RNA was isolated using the Trizol Reagent according to the manufacturer's instruction (Invitrogen, Carlsbad, California, USA) and used to synthesize the first strand of cDNA using the cDNA Synthesis Kit (TOYOBO Co., Osaka, Japan). To produce dsRNA for RNAi, PCR fragments were amplified by reverse transcription using the corresponding cDNA as template. The primers containing the T7 promoter sequence of *BmEcR*, *BmBR-C* and *BmE74A*, are shown in Table 1. dsRNAs of *BmEcR*, *BmBR-C* and *BmE74A* were synthesized using T7 RNA polymerase in the Ambion MEGAscript RNAi Kit (Ambion, USA) according to the manufacturer's instruction.

Table 1 Primers used in dsRNA synthesis

Gene	GenBank accession no.	Primer sequence (5′-3′)
BmEcR	D43943/L35266/ L35266	F: GAATTAATACGACTCACTATAGGGCTTTACCAATGCCTCCCA
		R: GAATTAATACGACTCACTATAGGGCGAAAGATACCTCGG GAC
BmBR-C	AB166730	F: GAATTAATACGACTCACTATAGGGGCAACCCTACCCTGACAA
		R: GAATTAATACGACTCACTATAGGGCGACTCCACATCCGATAC
BmE74A	DQ471939	F: GAATTAATACGACTCACTATAGGGCGACGACATCCTGAAGCA
		R: GAATTAATACGACTCACTATAGGGCGACTCCACATCCGATAC

The T7 promoter sequence is underlined.

Larvae at 1 d after wandering were used for dsRNA injection. Four micrograms of dsRNA were injected into the 7th segment of the abdomen of the larvae using a microinjector. Vaseline was applied to avoid the leakage of body fluids. Four injection groups including *BmEcR* dsRNA, *BmBR-C* dsRNA, *BmE74A* dsRNA and RNA elution buffer (negative control) were performed. At least 100 larvae were injected for each group. After dsRNA injection, the larvae were immediately divided into two sub-groups randomly and reared to adults for qPCR detection (40 larvae per sub-group) and morphological observation (60 – 75 larvae per sub-group).

2.3 Real-time PCR detection

For each injection (*BmEcR* dsRNA, *BmBR-C* dsRNA, *BmE74A* dsRNA and RNA elution buffer), forty larvae were randomly divided into three sub-groups for sampling. Three larvae were selected randomly from each sub-group and their posterior silk glands were dissected for qPCR at 1 d post pupation. Three dsRNA injection repeats were conducted for each of the data.

2.4 Morphological observation

For each RNAi treatment (*BmEcR* dsRNA, *BmBR-C* dsRNA, *BmE74A* dsRNA and RNA elution buffer), 60 – 75 larvae were randomly divided into three sub-groups for morphological observation. Developmental abnormalities, including failure to pupate or emerge, deviant wing and death rate, were recorded.

2.5 Analysis of quantitative real-time PCR (qPCR)

Total RNA was isolated using Trizol Reagent according to the manufacturer's instruction (Invitrogen, Carlsbad, California, USA) and used to synthesize the first strand cDNA according to the cDNA Synthesis Kit (TOYOBO Co., Osaka, Japan). The primers used for qPCR were designed based on the sequences of the corresponding target genes. For *BmEcR*, the primers were designed based on the conserved region of the three isoforms *BmEcR-1*, *BmEcR-2-1* and *BmEcR-2-2*. For *BmBR-C*, the primers were designed based on the conserved region of the three isoforms *BmBR-C-Z1*, *BmBR-C-Z2* and *BmBR-C-Z4*. For the transcription factor genes *BmE74B*, *BmFTZ-F1*, *BmHR39*, *BmE75B*, *BmIAP*, the primers were designed based on the specific regions of these genes. *BmActinA3* was used as the control. Primer sequences are shown in Table 2. qPCR was performed according to SYBR-Green fluorescent relative quantitative approaches (Livak and Schmittgen, 2001) in an ABI7300 apparatus. Three independent repeats were conducted for each of the data points.

2.6 Data analysis

The data analysis in the study was carried out using SPSS statistical analysis software. Significance test including the phenotype changes and changes in expression of related genes between injection group

and RNA elution buffer group (negative control group), was analyzed with *t*-test.

Table 2　Primer sequences used in qPCR

Gene	GenBank accession no.	Primer sequence (5′-3′)
BmEcR	D43943/L35266/L35266	F: TCTTCGGCACTGGGTTTG
		R: GGTGTTGTGGGAGGCATT
BmBR-C	AB166730	F: TGCCCTTCCAACCCTGAT
		R: TGAGCCAGTGCCTCCATT
BmE74A	DQ471939	F: GCCACCATCATCACAACAAA
		R: TTCAGCAGGAACTCCCACA
BmE74B	DQ471940	F: CACCACGTACCTGTGGGAGT
		R: TTCATGTCTGGCTTGTTC
BmFTZ-F1	D10953	F: GGTGAACCAGGAGCGTAT
		R: TGCCCACATCAAGACATT
BmHR39	AB005660	F: CCCGACATTCCACCTTTA
		R: CCGAGTTTGGCACTTTGA
BmE75B	AB024905	F: ATGGTGCGAACCATGTCG
		R: ACCGATTCCTGTTAATCCTGAG
BmIAP	AY155274	F: GGTGAAAGGACGTGACTACAT
		R: CGCTCCTCGGAATAACATA
BmActinA3	NM_001126254.1	F: CGGGAAATCGTTCGTGAT
		R: ACGAGGGTTGGAAGAGGG

3　RESULTS

3.1　Effect of RNAi on expression of the target genes *BmEcR*, *BmE74A* and *BmBR-C*

Expression of *BmEcR*, *BmE74A* and *BmBR-C* appeared earlier than those of the transcription factor genes *BmHR3A*, *BmHR39*, *BmβFTZ-F1*, *BmE74B*, *BmE75B* and *BmE75C* and these three genes might be at the up-stream of the ecdysone signal transduction pathway for programmed cell death (PCD) initiation in the silk gland (Li *et al.*, 2010). To examine whether the interference of *BmEcR*, *BmE74A* and *BmBR-C* influences the expression of the above transcription factor genes during metamorphosis, dsRNAs of these genes were respectively injected into the wandering larvae. The results indicated that the expression levels of *BmEcR*, *BmE74A* and *BmBR-C* were significantly ($P < 0.01$) decreased after RNAi treatments and were only 18.2%, 11.0% and 16.0% of the mock control, respectively (Fig. 1).

3.2　Morphological changes in response to RNAi of *BmEcR*, *BmE74A* and *BmBR-C*

The larvae injected with dsRNA of *BmEcR*, *BmBR-C* or *BmE74A* appeared to be asynchronous in pupation and eclosion, as compared to the control group. Among the 60-75 larvae injected, 4-8 larvae failed to pupate normally (Fig. 2: A, C, E)

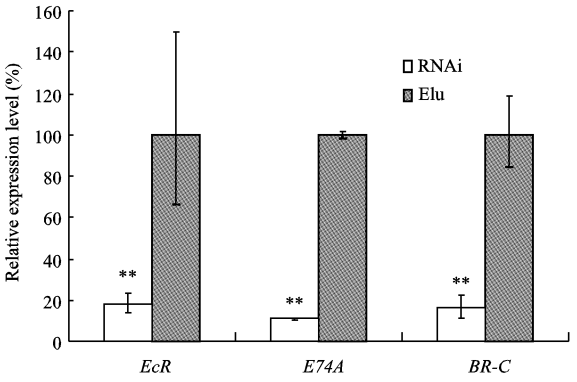

Fig. 1　q-PCR analysis of mRNA expression of *BmEcR*, *BmE74A* and *BmBR-C* genes suppressed by RNAi in *Bombyx mori*

Four μg of dsRNA or buffer alone was injected into the hemolymph of larvae at the wandering stage and total RNA was extracted at 1 d post pupation (about 5 d post RNAi treatment). The data are the mean ± *SE* of three independent experimental repeats. ** Statistically significant difference (*t*-test, $P < 0.01$).

and 5-8 moths appeared to be winglet (or deviant wing) after emergence from the cocoons (Fig. 2: B, D, F) in each treatment. Table 3 shows the statistics of the effect of the *BmEcR*, *BmBR-C* and *BmE74A* RNAi on the development of the treated *B. mori*. The rates of failure to pupate or emerge in *BmEcR*, *BmBR-C* and *BmE74A* RNAi treatments were 12.2%, 6.8% and 9.3%, respectively ($P < 0.05$); and the rates of winglet were 8.7%, 10.0% and 10.7%, respectively ($P < 0.05$); the rates of mortality were 23.0%, 13.3% and 22.7%, respectively ($P < 0.05$). The total rates of abnormality in *BmEcR*, *BmBR-C* and *BmE74A* RNAi treatments were 43.2%, 30.0% and 42.7%, respectively, being significantly higher than the control ($P < 0.01$).

3.3　Effect of RNAi of *BmEcR*, *BmE74A* and *BmBR-C* on expression of other transcription factor genes

Effects of *BmEcR*, *BmE74A* and *BmBR-C* RNAi on the expression of the ecdysone receptor gene *BmEcR*, the other transcription factor genes *BmE74A*, *BmBR-C*, *BmE74B*, *BmHR3A*, *BmβFTZ-F1*, *BmE75B* and *BmHR39*, and the apoptosis inhibitor protein gene *BmIAP* were examined (Fig. 3). While injection of *BmEcR* dsRNA caused a significant decrease of the *BmEcR* mRNA level (Fig. 1), the expression levels of the transcription factor genes *BmE74A*, *BmE74B*, *BmHR3A* and *BmβFTZ-F1*, were also significantly decreased ($P < 0.01$), whereas the expression of *BmBR-C*, *BmE75B* and *BmHR39* stayed at relatively stable levels (Fig. 3: A). This result indicated that *BmEcR* might be at the most up-stream of the genes *BmE74A*, *BmE74B*, *BmHR3A* and *BmβFTZ-F1* and was critical for the initiation of the ecdysone signal cascade. *BmE74A* RNAi significantly decreased the

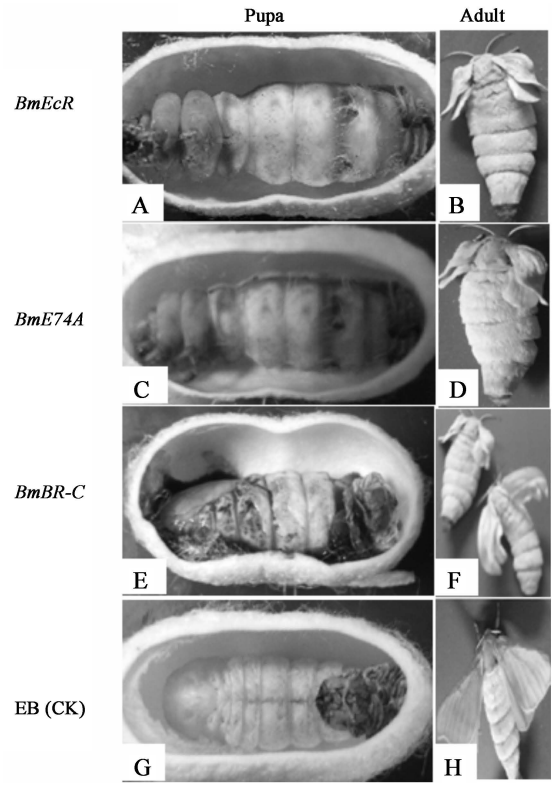

Fig. 2 Effects of RNAi of *BmEcR* (A, B), *BmE74A* (C, D), *BmBR-C* (E, F) and elution buffer (G, H) on the phenotype of *Bombyx mori*

dsRNAs were injected into the larvae at 1 d after wandering stage and the photographs were taken at 1 d post pupation (A, C, E, G) and 1 d post emergence (B, D, F, H). A, C, E: Abnormal pupae; B, D, F: Adults with winglets; G, H: Buffer alone; G: Normal pupa; H: Normal adult. EB: Elution buffer.

expression of *BmE74A* (Fig. 1), and also significantly suppressed the expression of *BmEcR*, *BmBR-C*, *BmE74B*, *BmHR3A* and *BmβFTZ-F1* ($P < 0.01$), but the expression of *BmE75B*, *BmHR39* and *BmIAP* was slightly increased (Fig. 3: B). RNAi of *BmBR-C* caused a significant decrease in the expression of all tested transcription factor genes except *BmIAP* (Fig. 3: C) ($P < 0.01$).

BmE74A is an earlier transcription factor of ecdysone signaling pathway (Fletcher *et al.*, 1996), and it should not regulate the transcription activity of ecdysone receptor BmEcR. However the transcription activity of *BmEcR* was significantly down-regulated after RNAi of BmE74A (Fig. 3: B). This result remains to be further verified.

4 DISCUSSION

4.1 *BmEcR*, *BmE74A* and *BmBR-C* might be the up-stream factors of the ecdysone signal transduction pathway in the silk gland

BmEcR, *BmE74A* and *BmBR-C* are the earlier expression genes in the silk gland during the larval to pupal transition in *B. mori* (Li *et al.*, 2010), and it is supposed that they might be at the up-stream of the ecdysone signal transduction initiating PCD during the degeneration of silk glands. In this study, RNAi not only suppressed the expression of the target genes *BmEcR*, *BmE74A* and *BmBR-C*, but also suppressed the expression of the other transcription factor genes *BmE74B*, *BmHR3A*, *BmHR39* and *BmE75B*,

Table 3 Influence of RNAi of *BmEcR*, *BmBR-C* and *BmE74A* on metamorphosis in *Bombyx mori*

Treatments	Number of larvae per group	Failure to pupate/emerge (%)	Deviant wing (%)	Mortality (%)	Total abnormality (%)
	22				
BmEcR	20	12.2 ± 1.1 *	8.7 ± 1.3 *	23.0 ± 3.6 **	43.2 ± 2.0 **
	23				
	20				
BmE74A	20	6.8 ± 1.7 *	10.0 ± 2.9 *	13.0 ± 1.7 *	30.0 ± 2.9 **
	20				
	25				
BmBR-C	25	9.3 ± 1.3 *	10.7 ± 1.3 *	22.7 ± 1.3 **	42.7 ± 1.3 **
	25				
	25				
Elution buffer	25	1.3 ± 1.3	1.3 ± 1.3	5.3 ± 1.3	8.0 ± 0.0
	25				

The values are mean ± *SE* (n = 3). Total abnormality rates are the sum of the numbers of failures to pupate or emerge, deviant wing and mortality divided by the total number (60 – 75) of the insects treated. * Significant difference at $P < 0.05$ between the treatment group and the elution buffer group (*t*-test); ** Significant difference at $P < 0.01$ between the treatment group and the elution buffer group (*t*-test).

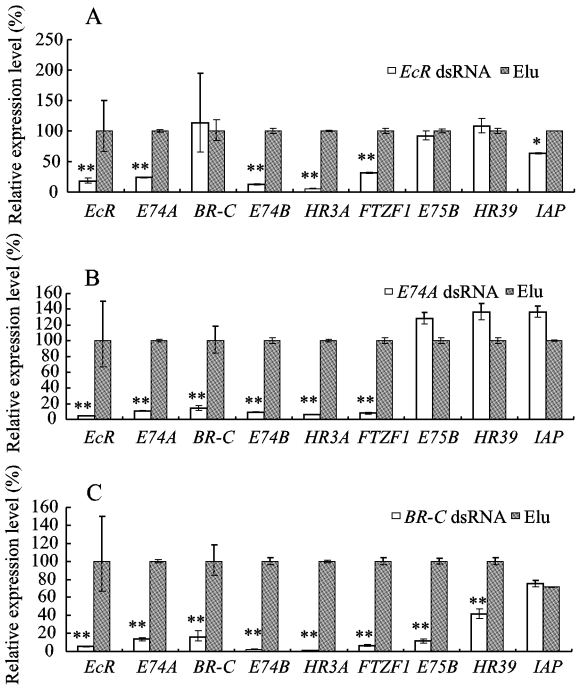

Fig. 3　Effects of RNA interference on the expression of ecdysone-related genes in *Bombyx mori*

Effect of injection with dsRNAs of *BmEcR* (A), *BmE74A* (B) and *BmBR-C* (C) on the expression of the ecdysone receptor gene *BmEcR*, the transcription factor genes *BmE74A*, *BmE74B*, *BmE75B*, *BmHR39*, *BmHR3A*, *BmFTZ-F1* and *BmBR-C*, and the apoptosis inhibitor protein gene *BmIAP*. The control was injected with elution buffer (Elu). The relative expression levels were detected by using qPCR and are represented as percentages over the control. ** Significant difference ($P < 0.01$) between the treatment and control, calculated using *t*-test. Four μg of dsRNA or buffer alone was injected into the hemolymph of 5th instar larvae at the first day of the wandering stage and the total RNA was extracted at day 5 post RNAi treatment. The data are the mean ± *SE* of three independent experimental repeats.

indicating that these three genes may function at the earlier phase during the PCD triggered by ecdysone. At the same time, the partial silence of these three genes by RNAi led to the failure of transition from larval to pupal stage or the later adult morphogenetic defects. Similar results were reported in literatures. Morphological changes of the anterior silk glands of *B. mori* were accompanied by the localization and expression of *BmEcR-B1*, indicating that *BmEcR-B1* expression is important for the PCD in the anterior silk glands (Goncu and Parlak, 2008, 2009). The degeneration of the larval silk glands from larvae to pupae was caused by autophagy and apoptosis, which were initiated by ecdysone signals at the later stage of 5th instar or wandering stage (Li *et al.*, 2010). Thus, we suggest that *BmEcR*, *BmBR-C* and *BmE74A* may act as the up-stream factors in the ecdysone signal transduction pathway and may be involved in the onset of either autophagy by activating the autophagy-related genes (*Atgs*) or apoptosis by activating the apoptosis-related caspase genes (Lee and Baehrecke, 2001; Baehrecke, 2003; Martin and Baehrecke, 2004; Li *et al.*, 2010).

In this study, the injection with RNA elution buffer was used as the control, and the study could be improved by using dsRNA of non-target gene of metamorphosis such as GFP as additional control. However, the data obtained from this study could provide basic information for further study.

4.2　*BmBR-C* might be regulated not only by the ecdysone but also other hormone or factors

The partial silence of *BmBR-C* by RNAi reduced the expression levels of *BmEcR*, *BmE74A*, *BmE74B*, *BmHR3A* and *BmβFTZ-F1* (Fig. 3: C). This result confirmed that *BmBR-C* may function at the earlier phase of the ecdysone cascade and regulate the expression of ecdysone receptor in the silk gland during the transition from larva to pupa (Karim *et al.*, 1993). However, the partial silence of *BmEcR* had no effect on the expression of *BmBR-C* (Fig. 3: A). This was possibly due to the changes of ecdysone and juvenile hormone (JH) during the period from Day 6 5th instar to earlier stage of pupa. Muramatsu *et al.* (2008) reported that the titers of ecdysone and JH increased obviously during the period from Day 6 d 5th instar to Day 9 5th instar; from Day 9 5th instar to pupation, ecdysone level showed a sharp decline after the titer of JH decreased rapidly to a very low level, but ecdysone increased sharply. The time of RNAi injection and sampling for qPCR at the experiment was Day 7 d 5th instar and 1 d post pupation, respectively, when the titers of ecdysone and JH remained high relatively. Therefore, it may be supposed that JH should antagonize the influence of ecdysone, which might neutralize the effect of *BmEcR* RNAi on the expression level of *BmBR-C*.

Zhou *et al.* (1998) reported that JH reduced *BR-C* expression to prevent metamorphosis in *Manduca sexta*. The *BR-C* expression was also controlled by JH in insects (Dubrovsky, 2005). In addition, Karim *et al.* (1993) proposed that the transcription factor BR-C played a key role in the stage-specificity of the ecdysone response in *Drosophila* and the ecdysone-receptor protein complex alone was not sufficient for appropriate induction of the early primary-response genes, the prior expression of BR-C proteins was definitely required. Consistent with the former studies (Karim *et al.*, 1993; Muramatsu *et al.*, 2008), our experimental data supported that *BmBR-C* might be regulated not only by the ecdysone but also by other hormone or factors.

However, some questions, such as what are the roles of *BmBR-C* in the hormone regulation of the *B. mori*, why suppressing the expression of *BmE74A*

and *BmBR-C* caused the decrease of the *BmEcR* expression, still need to be further investigated.

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RNA 干扰对家蚕丝腺蜕皮激素相关基因表达的影响

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摘要：昆虫变态发育过程中，蜕皮激素通过一系列的激素相关转录因子进行信号的转导和放大，从而完成对生长变态发育的调控，其中蜕皮激素受体(EcR)及转录因子 BR-C 和 E74A 可能作为早期因子发挥作用。为了研究这 3 个早期转录因子在鳞翅目昆虫中的功能，本研究采用体外合成 dsRNA 的方法，将合成的 dsRNA 分别注射熟蚕期的家蚕 *Bombyx mori*，进行 RNA 干扰，并对这 3 个基因被 RNA 干扰后的形态变化和相关的转录因子基因 *BmHR3A*，*BmHR39*，*BmβFTZ-F1*，*BmE74B* 和 *BmE75B* 的表达进行了实时荧光定量 PCR 检测。形态学观察结果显示，这 3 个基因被 RNAi 后出现异常的蛹和蛾、小翅和死蛹等表型。qPCR 检测结果表明：RNAi *BmEcR* 后导致了 *BmE74A*，*BmE74B*，*BmHR3A* 和 *BmβFTZ-F1* 表达水平的显著降低($P<0.05$)，RNAi *BmE74A* 后导致了 *BmEcR*，*BmBR-C*，*BmE74B*，*BmHR3A* 和 *BmβFTZ-F1* 表达水平的显著降低($P<0.01$)，而 RNAi *BmBR-C* 后则导致所有被检测的蜕皮激素受体及其相关转录因子的 mRNA 水平都显著降低($P<0.01$)。据此推测，*BmBR-C* 可能在蜕皮激素信号的早期发挥作用，并对其他相关转录因子和蜕皮激素受体有调控作用。

关键词：家蚕；RNA 干扰；变态；蜕皮激素；转录因子

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